REMARKS

Claims 1-5 are currently pending in the application. Claims 1, 3, 4, and 5 have been amended. Support for these amendments can be found throughout the specification and claims as originally filed.

Accordingly, no new matter has been added by the current amendments. Moreover, the claim amendments requested herein should in no way be construed as acquiescence to any of the rejections and have been made solely to expedite prosecution of the application. Applicants reserve the right to pursue the claims as originally filed and/or prior to amendments made herein in this or a separate application(s).

Rejections under 35 U.S.C. §102(e)

Applicants thank the examiner for withdrawal of the rejection of claims 1 and 3-5 as being anticipated by Sayegh, *et al.* (U.S. Patent No. 6,280,957).

Rejections under 35 U.S.C. §103(a)

Claims 1and 3-5 have been rejected as obvious over deBoer, *et al.* (U.S. Patent No. 5,869,050), in view of Lenschow, *et al.* (*Transplantation* (1995) 60:1171), Tarumi, *et al.* (*Transplantation* (1999) 67:520) and/or Newell, *et al.* (*J. Immunol.* (1999) 163:2358), and in further view of Chen, *et al.* (*Transplantation* (1995) 59:1084-1089), Strom, *et al.* (*Therapeutic Immunology* edited by Austen, *et al.* Blackwell Science, Cambridge, MA, (1996) pages 451-456) and Li, *et al.* (*Transplantation* (1998) 66:1387-1388; 1449, #B3).

More specifically, the Office Action states:

> Given that CTLA4IG blocks both B7-1 and B7-2-mediated responses and given the combination of anti-B7-1 and anti-B7-2 antibodies achieve significant inhibition of allogenic responses and graft rejection, one of ordinary skill in the art would have been motivated to combine both B7-1-specific and B7-2-specific antibodies to inhibit transplant rejection, including intestinal rejection. Given the teachings of deBoer, *et al.*, Lenschow, *et al.*, Tarumi, *et al.*, and Newell, *et al.*, the ordinary artisan would have an expectation of success in prolonging intestinal graft survival by blocking both B7-1 and B7-2 mediated interactions. Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Applicants respectfully traverse this rejection, and submit that, at most, the prior art references cited in the Office Action merely provide a general suggestion to try various combinations of immunosuppressive antibodies and immunosuppressive agents. However, this is not the standard required to establish obviousness under 35 U.S.C. §103. See *In re Dow Chemical Cp.,* 837 F.2d 469 (Fed. Cir.1988). "'Obvious to experiment' is not a proper standard for obviousness". And see *In re Eli Lilly & Co.,* F.2d 943 (Fed. Cir. 1990) "An 'obvious to try' situation exists when a general disclosure may pique the scientist's curiosity, such that further investigation might be done as a result of the disclosure."

Specifically, the Office Action maintains that the combined teachings of deBoer, *et al.* and Lenschow, *et al.* provide "sufficient motivation and expectation of success in combining the combination of anti-B7-1 and anti-B7-2 antibodies plus rapamycin to downmodulate the immune response to an intestinal allograft in a subject" (Office Action at page 3).

Contrary to this assertion, Applicants respectfully point out that deBoer, *et al.* teach that T cell activation can be inhibited in a tissue culture assay using a combination of anti-B7-1 antibodies and cyclosporine A. Based on this single *in vitro* exemplification, de Boer *et al.* then propose that transplant rejection, in general, may be treated *in vivo* using a combination of anti-B7-1 antibodies and an immunosuppressive agent. Lenschow, *et al.* teach that the combination of anti-B7-1 and anti-B7-2 mAbs significantly prolongs the mean survival time of allografts of pancreatic islet cells beyond the use of CTLA4Ig or anti-B72 alone. Accordingly, the combined teachings of these two references, at best, merely teach that anti-B71 antibodies may be combined with certain second immunosuppressive agents (e.g., cyclosporine A or anti-B72 antibodies) to inhibit T cell activation *in vitro* and/or to treat transplant rejection of pancreatic islet cells, and further that combination of anti-B71 and anti-B72 antibodies is more effective tha CTLA4Ig. However, neither deBoer, *et al.* or Lenschow, *et al.* teach or suggest the combination of B7-1 and B7-2 antibodies *and a third immunosuppressive* agent to treat any kind of allograft, let alone the combination of B71 and B72 antibodies plus rapamycin to treat an intestinal allograft as presently claimed.

As previously presented by Applicants, and reiterated in full herein by reference to Applicants' previous response, the nature of the immune response to intestinal allografts is unique compared to other types of allografts. Thus, the skilled artisan readily understood that

the results obtained from *in vitro* experiments (as taught by de Boer *et al.)* or with other types of allografts (such as pancreatic islet cells as taught by Lenschow, *et al.*) cannot be extrapolated to transplantation of intestinal grafts with any reasonable expectation of success. Specifically, the state of the art at the time of filing recognized that therapies which are effective at preventing transplant rejection of allografts of other tissues are ineffective in the prevention of intestinal allograft rejection.

Indeed, a recent publication by Newell, K.A. (*Am. J. Transplantation* (2003) 3:1-2, Appendix A) provides additional evidence that it is generally recognized that intestinal transplants are different from the transplantation of other organs, and that the intestine is a particularly immunogenic organ. For example, at page 1, 1st paragraph, left column, Newall, *et al.* states that "[b]oth clinical and experimental evidence demonstrate significant differences in the immunogenicity of different organs. For example, while rejection rates of approximately 10% are now widely reported following kidney transplantation, ***rejection remains the rule following intestinal transplantation***." Newell (2003) also presents an explanation for the differing immunogenicity of various organs. At page 1, 2nd paragraph, left column, Newell states, "[w]hile CD8+ T cells are neither necessary nor sufficient to cause rejection of cardiac allografts, they are fully capable of independently mediating the rapid rejection of intestinal allografts."

Moreover, Applicants submit that cyclosporine (CsA) (as taught by deBoer, *et al.*) and rapamycin are not equivalent molecules. Cyclosporine is an immunosuppressive agent that binds with high affinity to the peptidyl-prolyl cis-trans-isomerase, Cyclophilin (CYPH), which may act to expose DNA binding sites required for the activation of some cytokines. Rapamycin is an immunosuppressive macrolide antibiotic that acts by inhibiting the mammalian targert of rapamycin (mTOR), a serine-threonine kinase. Kahan (2001, *Expert Opin Pharmacother*, 2:1903, Appendix B). Thus, one skilled in the art would recognize that rapamycin and cyclosporine cannot be used interchangeably since they operate through very different cellular mechanisms, and thus the successful substitution of rapamycin in the teachings of deBoer, *et al.* is not predictable.

Accordingly, the teachings of deBoer, *et al.* or Lenschow, *et al.* taken either separately or in combination do not teach or provide the motivation to one skilled in the art that the claimed combination of an anti-B7-1 antibody, an anti-B7-2 antibody and a rapamycin compound could

effectively treat or prevent any type of allograft rejection, let alone rejection of an intestinal allograft.

Furthermore, although the Office Action continues and states that "Tarumi, *et al.* and Newell, *et al.* clearly provided sufficient motivation and expectation of success in targeting B7-1 and B7-2 in the transplantation of intestinal allografts," these references do not adequately compensate for the deficiencies of the disclosures of deBoer, et al. and Lenschow, *et al.*, nor do they provide the motivation lacking in the teachings of deBoer, *et al.* and Lenschow, *et al.* to use a combination of three molecules, anti-B71, anti-B7-2 and rapamycin, as presently claimed. Indeed, Newell, *et al.* (1999) teaches that administration of CTLA4-Ig (which binds B7-1 and B7-2) is ***insufficient*** to block intestinal allograft rejection in wild type mice, and Tarumi, *et al.* confirm the teachings of Newell, *et al.* (1999), thus teaching away from the present invention.

Moreover, Applicants again point the Examiner to Newell (2003) in support of their position that CTLA4-Ig is not equivalent to B7-1 and B7-2, and that one skilled in the art would not have had a reasonable expectation of success in substituting CTLA4-Ig with anti-B7-1 and anti-B7-2 antibodies to prevent intestinal allograft rejection. More specifically, Newell (2003) states that "[c]ostimulation blockers, such as CTLA4-Ig, that effectively control alloreactive CD4+ T cells, but not alloreactive CD8+ T cells, prevent the rejection of heart, but not intestinal allografts in mice (citing references 7-9)." (page 1, 1st paragraph, left column). This difference between treatment of intestinal allografts with CTLA4-Ig and B7-1 and B7-2 antibodies is also demonstrated in the instant specification which teaches that "[i]nhibition of the CD28 pathway by anti-B7 mAbs or by genetic disruption blocks intestinal allografts more effectively than does mCTLA4Ig suggesting that not all approaches to blocking a costimulatory pathway are equivalent." (see page 40, lines 4-6, Example 1, pages 38-40, and Table 1, page 40). Therefore, although one skilled in the art may be motivated with a reasonable expectation of success to treat a cardiac allograft with CTLA4-Ig to prevent rejection, one skilled in the art would ***not*** be motivated and would not have a reasonable expectation of success to treat an intestinal allograft with CTLA4-Ig since the innate immunity of intestine is significantly different than the heart.

The Office Action further cites Chen *et al.*, Strom *et al.*, and Li *et al.* as providing further support that the motivation and expectation of success for the presently claimed invention existed in the combined teachings of the cited references. Specifically, the Office Action states that Chen, *et al.* "teach rapamycin pretreatment prolongs small bowel transplantation (see entire

document, including Abstract and Discussion). Therefore, the immunosuppressive rapamycin has been shown to have particular effects with respect to the survival of intestinal allografts," that Strom *et al.* teach "in therapeutic approaches to organ transplantation that several agents are used simultaneously, each of which is directed at a different molecule target," and that Li *et al.* "teach all host treated with rapamycin and costimulation blockade (e.g., CTLA4Ig) achieved permanent engraftment in contrast to the use of cyclosporine as the immunosuppressive regimen" (Office Action at pages 4-5).

Applicants submit that the teachings of Chen, *et al.*, Strom, *et al.* and Li, *et al.* do not cure the deficiencies of deBoer, *et al.* or Lenshow, *et al.* in view of Tarumi, *et al.* and/or Newell, *et al.* (1999). Chen, *et al.* teach that pretreatment of a donor intestinal graft with rapamycin in combination with treatment of the recipient with cyclosporine can prolong recipient survival. However, Chen, *et al.* do not teach or suggest the use of a combination of anti-B7-1, anti-B7-2 and rapamycin in preventing intestinal allograft rejection as presently claimed.

Strom *et al.* generally teach a multi-tiered approach to immunosuppressive therapy. Strom *et al.* further disclose that the majority of basic protocols involve a combination of cyclosporine or Fk506 plus corticosteroids with or without azathioprine, and suggest that anti-lymphocyte globulin or OKT3 might also added to reduce the dose of cyclosporine required (page 454). Thus, while Strom *et al.* do include rapamycin in their general description of immunosuppressants, they do not actually teach or suggest the use of rapamycin in any multi-tiered immunosuppressive therapy regimen.

Li, *et al.* teach that a combination of costimulation blockade and rapamycin treatment produce permanent engraftment of ***cardiac allografts***. However, Li, *et al.* do not teach or suggest the use of a combination of anti-B7-1, anti-B7-2 and rapamycin in preventing intestinal allograft rejection as presently claimed. Furthermore, as discussed above as taught by Newell (2003), successful treatment to prevent rejection of cardiac allografts is not predictive of the same treatments' ability to prevent the rejection of intestinal allografts because of the significantly different immunogenicity of an intestinal allograft.

In short, Applicants submit that none of the cited references alone or in combination suggest the use of a combination of rapamycin with anti-B7-1 and anti-B7-2 antibodies as presently claimed to inhibit intestinal allograft rejection. Indeed, Applicants submit that the

Examiner has failed to point to any teaching in the deBoer, *et al.*, Lenschow, *et al.*, Tarumi, *et al.*, Newell, *et al.*, Chen, *et al.*, Strom, *et al.*, and Li, *et al.* references that would compel one of ordinary skill in the art to make the claimed invention. The prior art must suggest "to those of ordinary skill in the art that they ***should*** make the claimed composition or device, or carry out the claimed process" and [b]oth the suggestion and the reasonable expectation of success ***must be founded in the prior art, not in the applicant's disclosure*** (emphasis added)." *In re Dow Chemical Co.* 837 F.2d 469, 473, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988).

In light of the above, Applicants maintain that the Examiner has not only failed to demonstrate that the motivation to combine the cited references existed at the time of the invention, by has also failed to demonstrate that their combined teachings provide a reasonable expectation of success to the ordinary skilled artisan at the time the invention was made. Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

SUMMARY

In view of the remarks set forth above, it is respectfully submitted that this application is in condition for allowance. It there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicant's attorney at (617) 227-7400.

Dated: September 9, 2004

Respectfully submitted,

By _____

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APPENDIX A

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Editorial

Transplantation of the Intestine: Is it Truly Different?

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Since Dr Joseph Murray performed the first kidney transplant that resulted in long-term success in 1954, transplantation has grown remarkably and now is considered optimal therapy for many diseases that result in failure of the kidney, liver, heart, pancreas, lung, and intestine. Interestingly, as transplantation has expanded to include new organs and as new immunosuppressive agents have been developed, only limited attempts to design organ-specific immunosuppressive strategies have been undertaken. This is despite the fact that both clinical and experimental evidence demonstrate significant differences in the immunogenicity of different organs (1,2). For example, while rejection rates of approximately 10% are now widely reported following kidney transplantation, rejection remains the rule following intestinal transplantation (3,4). In fact, the poorer outcomes associated with transplantation of the intestine are largely the result of the more frequent and severe rejection observed following intestinal transplantation and the complications of the intense immunosuppression required to control the immune response (3,5). When attempts to design organ-specific approaches to immunosuppression have been undertaken, by necessity their design has been empiric rather than being based upon an understanding of organ or tissue-specific mechanisms of allograft damage. Perhaps because the empiric approach to study design does not appeal to a transplant community grounded in scientific method, it remains common to use combinations of immunosuppressive agents to prevent the rejection of one type of transplanted organ that have been proven effective in clinical trials that studied a different type of transplanted organ.

One of the most likely explanations for the differing immunogenicity of various transplanted organs is that the mechanisms of immune-mediated allograft damage are qualitatively different. Numerous experimental models of transplantation provide evidence to support this hypothesis. One example is the differing role of CD8+ T cells in the rejection of murine cardiac and intestinal allografts. While CD8+ T cells are neither necessary nor sufficient to cause rejection of cardiac allografts (6), they are fully capable of independently mediating the rapid rejection of intestinal allografts (7). Differences in the relative roles of the mechanisms that contribute to the rejection of various transplanted organs may explain why some transplanted organs are more sensitive to a given immunosuppressive agent than are others. This principle is clearly illustrated by the variable effectiveness in murine transplant models of agents that block costimulatory pathways. Costimulation blockers, such as CTLA4-Ig, that effectively control alloreactive CD4+ T cells but not alloreactive CD8+ T cells, prevent the rejection of heart but not intestinal allografts in mice (7–9). Clinical evidence to support the concept of organ-specific differences in mechanisms of rejection is provided by the observation that different organs display a spectrum of sensitivity to humorally mediated rejection. While kidneys appear to be uniformly susceptible to damage mediated by preformed alloantibodies, liver allografts appear largely resistant to this type of immunologic damage. Hearts (and perhaps intestines based on data presented in this issue) appear to display an intermediate sensitivity to humoral rejection. It will be important to determine whether differences of this type represent a difference in the type of immune response initiated by an organ allograft or a difference in the susceptibility of different organs or tissues to a given effector mechanism. In either event, these observations provide a rationale for carefully studying each type of transplanted organ and caution against extrapolating from one type of transplanted organ to another.

In this issue of the *American Journal of Transplantation* Ruiz and colleagues describe the histologic appearance of a previously unrecognized pattern of allograft injury occurring in patients following intestinal transplantation. This pattern of injury was characterized by dilatation and erythrocyte congestion in the small venous and arterial vessels of the submucosa and edema and extravasation of erythrocytes within the interstitium. The authors noted that this pattern of injury was associated with a higher pretransplant percentage of panel reactive antibodies, a higher incidence of positive T and B cell crossmatches, and a higher incidence of graft loss as a result of rejection (all of which were statistically significant). Compatible with their observations of C4d and alloantibody deposition in allograft blood vessels, they conclude that this histologic pattern of injury represents antibody mediated acute vascular rejection. However, this acute vascular rejection is different from that observed after kidney transplantation in that it often occurs later following transplantation, and seems to be responsive, at least in some cases, to standard immunosuppressive interventions. An awareness of this

type of injury may improve the outcome of intestinal transplantation by triggering the earlier treatment of allograft rejection. In addition, further study may show that this pattern of rejection is amenable to treatment strategies not routinely used following intestinal transplantation. As important as the observations of Ruiz and colleagues may eventually prove to be, it should be emphasized that the findings of congestion in the submucosal vessels and edema with extravasation of erythrocytes in the interstitium are relatively nonspecific. The more specific findings of C4d and antibody deposition within the blood vessels are limited to a very small subset of the study population. Thus, it would be prudent to await additional studies before fully accepting the authors' conclusion that this histologic pattern of injury defines a new clinical entity. If, however, this interpretation of the data is confirmed, in addition to having important implications for the future of intestinal transplantation, the authors' findings will provide additional clinical evidence that reinforces the concept that different transplanted organs elicit different immune responses and should provide further impetus to development organ-specific immunosuppressive regimens.

Although perhaps not universally accepted and certainly not widely exploited in designing immunosuppressive regimens, the assertion that different transplanted organs elicit mechanistically distinct immune responses is consistent with and supported by substantial clinical and experimental data. Determining the clinical importance of this concept will require careful studies that compare and contrast the immune response of patients following transplantation with different types of organs. Central to these investigations will be the routine acquisition of biopsy material during episodes of allograft dysfunction. A thorough histologic and molecular study of the biopsy tissue should contribute to a more complete understanding of how the recipient immune system responds to different transplanted organs and how different transplanted organs are affected by a given type of recipient immune response. The findings of Ruiz *et al.* demonstrate how a policy of frequent allograft biopsy (the authors performed 188 biopsies in 21 patients within 3 months of transplantation) may provide new insights into the mechanisms of allograft damage following transplantation. The role of allograft biopsy has recently been emphasized in an editorial by Dr Phillip Halloran in which he discussed the etiology, evaluation, and treatment of chronic allograft nephropathy (10). I agree with Dr Halloran that allograft biopsy is a critical tool that offers great opportunity for developing a fuller understanding of the immune and nonimmune mechanisms that contribute to allograft damage. For this tool to be most effective, all episodes of organ dysfunction should be evaluated by biopsy. This is particularly true for highly immunogeneic organs such as the intestine.

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Expert Opinion

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Sirolimus: a comprehensive review

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Sirolimus (Rapamune®, Wyeth-Ayerst, Madison, NJ) is a new, potent, immunosuppressant that is emerging as a foundation for long-term immunosuppressive therapy in renal transplantation. The drug acts during both co-stimulatory activation and cytokine-driven pathways *via* a unique mechanism: inhibition of a multifunctional serine-threonine kinase, mammalian target of rapamycin (mTOR). Although there is no *a priori* reason to assume it, sirolimus displays a synergistic interaction to enhance the efficacy of cyclosporin A (CsA). In trials wherein the concentrations of CsA and sirolimus were tightly controlled, rates of acute rejection episodes were ≤ 10%, despite markedly reduced exposures to each agent. In pivotal multi-centre blinded dose-controlled trials, the rates of acute rejection episodes within 12 months following administration of 2 or 5 mg/day sirolimus in combination with CsA and steroids were reduced to 19 and 14%, respectively. Since the inhibitory effect of sirolimus disables virtually all responses to cytokine mediators due to the widespread involvement of mTOR in multiple signalling pathways, the agent is likely also to retard proliferation of endothelial and vascular smooth muscle cells, an important component of the immuno-obliterative processes associated with chronic rejection. The advantages of this unique therapeutic action combined with an intrinsic lack of nephrotoxicity are counterbalanced by myelosuppressive and hyperlipidaemic side effects. Ongoing studies are assessing whether the long-term benefits of sirolimus to permit reduction in exposure to or elimination of calcineurin inhibitors ameliorate the progression of chronic nephropathy, the condition that erodes long-term renal transplant survival.

Keywords: cytokines, immunosuppression, induction therapy, refractory rejection, sirolimus, steroid withdrawal

Expert Opin. Pharmacother. (2001) 2(11):1903-1917

1. Overview of the market

The potential scope of clinical immunosuppression cannot be accurately estimated, for the size of the market is critically dependent upon the therapeutic window (TW) of the agent between efficacy *versus* toxicity. That is to say, the relatively modest efficacy of the drug combination used initially for pharmacological immunosuppression - azathioprine (Aza) and prednisone (Pred) - barely outweighs the toxicity of the regimen. The long-term clinical results of cadaveric donor renal transplantation in the 1960s and 1970s were only acceptable in view of the hazards associated with chronic dialysis. In that era, one could only have remotely predicted that the number of transplants would increase more than 200-fold upon introduction of the more selective immunosuppressant CsA in 1983.

Rather than inhibiting elements of non-specific host resistance as was the case with Aza and steroids, the action of CsA is focused on calcium-dependent T- and probably B-lymphocyte pathways requiring activation of the phosphatase calcineurin. Introduction of CsA has increased the efficacy of immunosuppression, raising overall 1-year graft survival rates to 90.8% [1], producing less myelosuppression and permitting

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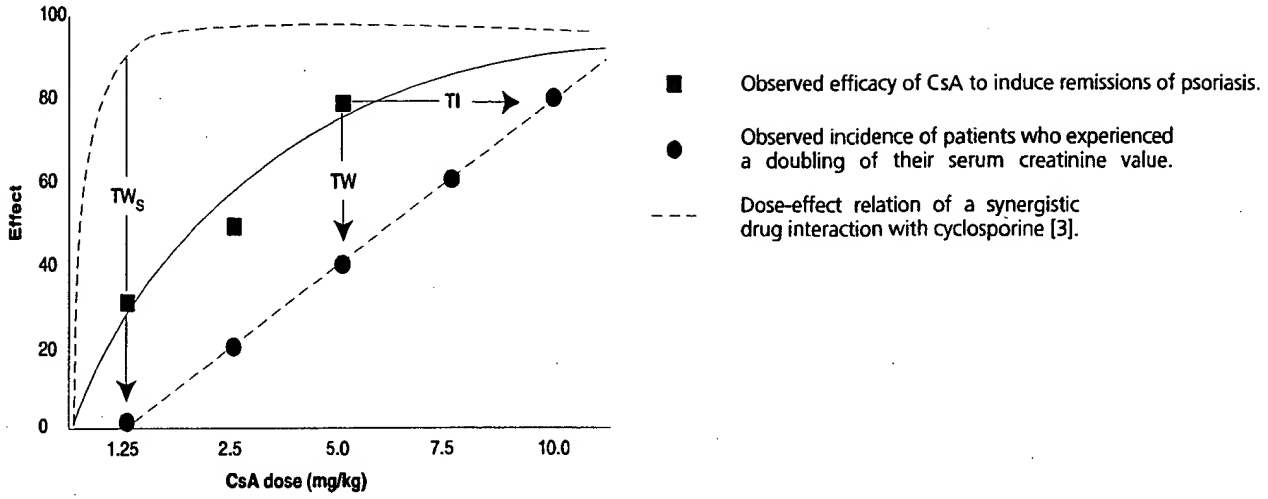

Figure 1. Potential impact of a second agent acting synergistically with CsA to broaden the therapeutic window of clinical immunosuppression. TI: therapeutic index, the quotient of the dose that produces toxicity and the dose that shows efficacy (TI = 2); TW: therapeutic window, the % immunosuppressive effect *versus* toxic effect at a given drug dose, namely, TW = 80/40 = 2. TWs: Therapeutic window of a synergistic drug combination, namely, TWs = 90/1.25 = 73.

the administration of reduced doses of debilitating corticosteroids. The impact of the introduction of CsA into this market cannot be overstated: it expanded the numbers of renal, liver and cardiac transplants, as well as the types of organ replacements to pancreas and lung. Using a more potent inhibitor of the same target enzyme calcineurin - tacrolimus - small bowel transplants are becoming increasingly successful as well.

However, the nephro- and neurotoxic limitations of both calcineurin antagonists have limited the extension of the use of these drugs into the settings of autoimmune diseases. For example, a 12-week course of CsA therapy (5.0 mg/kg) to patients with refractory psoriasis vulgaris was reported to induce an 80% remission rate [2] and a 40% incidence of doubling of the serum creatinine [3], yielding a TW of 2.0 (Figure 1). Although combination therapy with the nucleoside synthesis inhibitor mycophenolate mofetil (MMF) has added to the efficacy of CsA-based therapy, reducing the incidence of acute rejection episodes from 40% to about 25% [4], the agent permits only a modest reduction in calcineurin antagonist exposure.

An alternate approach has emerged following the introduction of a new agent, sirolimus. In studies comparing CsA-Aza-Pred to sirolimus-Aza-Pred [5] or CsA-MMF-Pred to sirolimus-MMF-Pred treatments [6], rates of acute rejection episodes of about 40% were similar in both arms. Pivotal trials revealed augmented efficacy when sirolimus was combined with CsA [7,8]. Furthermore, a median effect analysis of the pivotal trial data documented that the drug combination displays a synergistic interaction [9], permitting marked reduction of calcineurin antagonist exposure. The synergistic interaction broadens the TW of CsA efficacy *versus* nephrotoxicity from 2 to 73 (Figure 1). However, even though sirolimus is not nephrotoxic, simple conversion of immunosuppression from a CsA-sirolimus to a sirolimus-based therapy may not be optimal even after the time of the major risk of acute rejection because of the necessity for higher exposures to and hazards of hyperlipidaemic toxicities from the mTOR inhibitor. Thus, the author advocates ongoing treatment with the synergistic CsA-sirolimus combination, tailoring exposure to each drug based upon the individual patient's responses. Although sirolimus represents an important advance in immunosuppression, it is unlikely to afford a tremendous extension of immunosuppressive therapy to patients with autoimmune diseases until robust algorithms are developed that broaden the TW for hyperlipidaemia, which now replaces nephrotoxicity as the primary adverse effect of the regimen. In summary, the issues of potentially increased comorbidity, inconvenience of lifetime therapy and cost (in the US, US$1000 mg/year), all limit eventual market penetration of sirolimus despite its novel immunosuppressive activity.

2. Introduction to the compound

Sirolimus is a hydrophobic macrocyclic lactone isolated from *Streptomyces hygroscopicus*, a soil actinomycete recovered from the Vai Atari region of Easter Island [10]. Macrocyclic lactones are lipophilic molecules bearing a 12-, 14-, or 16-membered lactone ring substituted with hydroxyl, methyl, or ethyl groups, as well as carbonyl functions with one, two, or three carbohydrate fragments. Sirolimus (Figure 2) bears a structural

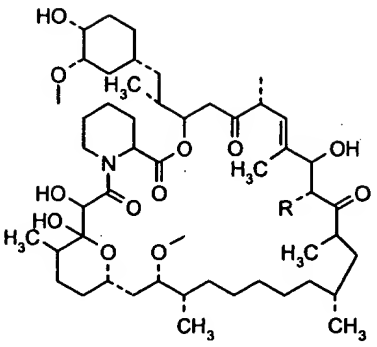

Figure 2. The chemical structure of sirolimus: (3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone.

resemblance to other macrocyclic lactones, such as tacrolimus and erythromycin. Although initial studies revealed anticandidal activity, commercial development was based upon the immunosuppressive potency of the drug originally discovered using experimental animal models of autoimmune diseases [11,12] and later of organ transplantation [13,14].

3. Pharmacodynamics

The hydrophobic drug readily penetrates the plasma membrane to attach to FK-binding protein 12 (FKBP 12) in the cytoplasm [15]. The sirolimus-FKBP 12 complex inhibits mTOR. This enzyme catalyzes distinctive reactions mediating the co-stimulatory cascade and the G_1 build-up following T- or B-cell activation. Sirolimus prevents activation of the inhibitory NF-κB kinase necessary for generation of the c-Rel transcription factors of the NF-κB complex [16] and possibly also modulates PKC activity [17]. During the subsequent G_1 phase triggered by the cytokine signal 3, sirolimus inhibits four signaling pathways:

- $p27^{kip1}$ degradation [18,19] leading to cyclin and kinase activation necessary for entry into the S phase [20,21]
- $p70^{S6}$ kinase phosphorylation, a step necessary for the synthesis of endosomal structural proteins [22,23]
- elongation factor (eIF) 4A release from its association with PHAS-I, thereby facilitating protein synthesis by ribosomes [24-26]
- transcriptional upregulation of the anti-apoptotic proteins bcl [27,28] and p21Ras [29] (Figure 3) [30]

In summary, sirolimus potentiates the inhibition produced by calcineurin antagonists during the signal 1 antigen-driven cascade [31] by acting on co-stimulatory signal 2 and by monoclonal antibodies (mAbs) that bind the distinctive CD25 component of the IL-2 receptor (IL-2R) [32] by acting on all cytokine signal transduction pathways (signal 3). The resulting matrix of CsA, anti-IL-2R mAbs and sirolimus collaborate in a selective immunosuppressive strategy denoted as the 'cytokine paradigm' (Figure 4) [33].

4. Pharmacokinetics and metabolism

Sirolimus, like the calcineurin antagonists CsA and tacrolimus, is a critical-dose drug [34]. Inappropriate drug doses can produce important consequences: namely, a rejection episode or drug-induced toxicities. Furthermore, sirolimus displays significant inter- and intrapatient variability that is poorly predicted by standard body measures, demographic features, or laboratory parameters, an observation that renders *a priori* selection of the optimal therapeutic dose impossible.

Concentration measurements are essential to optimise outcomes. Our study of 150 renal transplant recipients treated with a sirolimus-CsA ± Pred regimen followed over a period of 4 years demonstrated the value of serial measurements of parent compound sirolimus using a high-performance liquid chromatography (HPLC) procedure combined with ultraviolet (UV) detection [34]. This method meets the current standards of practice and performance with the flexibility and modest cost necessary to support the needs of a clinical transplant program [35]. Concentrations measured by HPLC/UV showed an excellent correlation with those resolved by tandem mass spectrometry [34,36], demonstrating that the HPLC/UV method selectively detects parent compound. Other assay methods such as the immunoassay (Abbot Imx; No. Chicago, Illinois) or a radioreceptor assay [37] display cross-reactivity with metabolites, which not only possess little biological activity [36,38,39], but also display variable and unpredictable intra- and interindividual variabilities [40].

The pharmacokinetic properties of sirolimus have been examined after a single dose, after a 2-week course and during maintenance therapy (Table 1) [41]. Sirolimus exhibits rapid GI absorption, a long terminal half-life ($t_{1/2}$) and extensive partitioning into formed elements of blood and tissues [42].

Of primary interest are the potential pharmacokinetic interactions between sirolimus and other drugs. Since sirolimus was initially approved by the FDA for use with full doses of the microemulsion formulation of CsA, it was critical to recommend spaced dosing of the two agents [43]. Simultaneous administration with full doses of CsA (6 - 10 mg/kg) markedly increased sirolimus exposure. The interactions included competition for metabolism by cytochrome P450 (CYP) 3A4 [44] and possibly for drug extrusion from the cell by P-glycoprotein [45]. In contrast, there was little drug interaction with sirolimus when administered concomitantly with the oil-based gel-cap formulation, Sandimmune (Zimmerman, personal communication) or with the currently recommended modest doses of CsA microemulsion (1 - 2 mg/kg/day; Kahan, unpublished observations) or with tacrolimus (2 - 5 mg/day) [46].

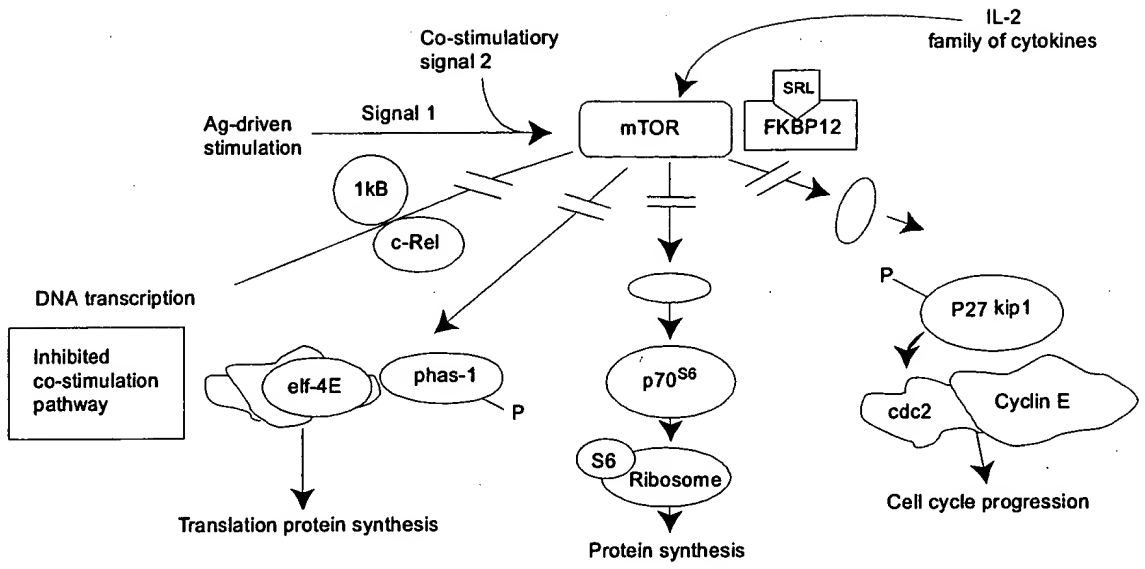

Figure 3. Sites of action of the multifunctional serine-threonine kinase mTOR both in the co-stimulatory pathway following antigen-driven activation and in the cytokine-driven stimulation pathway. Ag: Antigen; Elf: Elongation factor; FkBP: Fk-binding protein; G1: Growth phase of cell cycle; mTOR: Mammalian target of rapamycin.

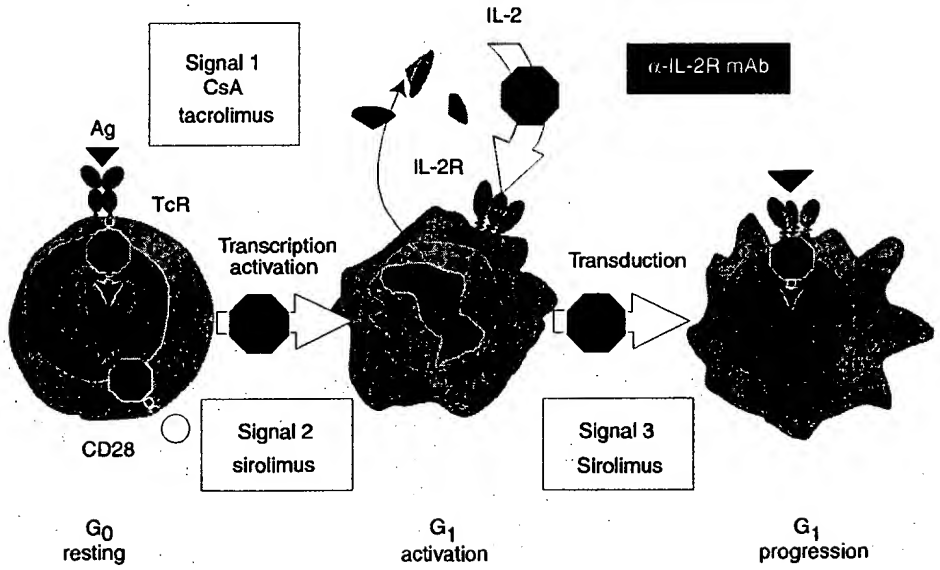

Figure 4. The cytokine paradigm: complementary sites of action of immunosuppressive drugs during lymphocyte activation. CsA and tacrolimus inhibit transcription of T-cell growth-promoting genes (e.g., IL-2). Anti-IL-2 mAbs block binding of IL-2 to its receptor. Sirolimus blocks growth-factor-initiated signal transduction. Ag: Antigen; G0: Resting phase of cell cycle; G1: Growth phase of cell cycle; mAb: Monoclonal antibody.

Table 1. Steady-state pharmacokinetic parameters of sirolimus in various patient populations.

Parameter	Value (patient population)
Absorption	
Median t_{max} (h)	≤ 1 (CV 86%) (All patients)
Oral bioavailability (%)	13.6 (95% CL, 10.3 - 16.9) (Stable renal transplants)
Distribution	
Blood:plasma ratios (mean ± SD)	36.5 ± 17.9 (Stable renal transplants)
Protein binding of plasma fraction (%)	92 (*in vitro*)
VD_{ss} (4 kg)	1.6 (CV 65%) (Stable renal transplants)
AUC correlation to $Cmin_{ss}$	0.96 (US multi-centre trial)
Metabolic pathway	CYP 3A4
Urine excretion (%)	2.2 (Healthy male volunteers)
Elimination $t_{1/2}$ (hours, mean ± SD)	59.2 ± 18.5 (CV 31%)
CL dependent variables	Paediatric age, hepatic impairment
Variability $Cmin_{ss}$	
Intra-individual % CV (g)	45 (US multi-centre trial)
Inter-individual % CV	38 (US multi-centre trial)
Interactions	
Food	Exposure increased
Cyclosporin, diltiazem, ketoconazole	Exposure increased
Rifamycin	Exposure decreased
Acyclovir, digoxin, nifedipine	Exposure unchanged

AUC: Area under the concentration-time curve; CL: Clearance; $Cmin_{ss}$: Trough concentration at steady state; CV: Coefficient of variation; $t_{1/2}$: Elimination half life; t^{max}: Time to maximal concentration; VD_{ss}: Volume of distribution at steady state. Reproduced with permission from KAHAN BD, CAMARDO JS: Rapamycin: Clinical results and future opportunities [Overview]. *Transplantation* (2001) **72**(8) (In Press) [41].

As a result of the potential impact of pharmacokinetic interactions to obfuscate pharmacodynamic synergy, rigorous median effect analyses had to be performed using drug concentrations rather than doses to dissect immunological from pharmacological events. For example, in the past, failure to recognise this principle led to the erroneous conclusion that MMF exerted synergistic interactions with sirolimus in rats [47]. The augmented effect compared with combination therapy with CsA was subsequently shown to reflect an effect of sirolimus to increase MMF concentrations [6,48], as has been previously observed with tacrolimus [49]. Concentration measurements revealed that CsA exerts an adverse effect both on MMF absorption and on metabolic recycling [50].

Sirolimus is manufactured as both an oral solution and a tablet. Owing to the importance of drug formulation on the pharmacokinetic profile and bioavailability of critical-dose drugs such as CsA [51], repetitive pharmacokinetic profiles were compared in 21 renal transplant recipients treated with the liquid form of sirolimus for more than 1 year before conversion to the tablet formulation [52]. These profiles showed similar area under the concentration-time curve (AUC) values between the formulations but lower maximal concentrations after tablet administration. Conversion from one formulation to another was associated neither with episodes of acute rejection nor with changes in laboratory values during the 8-week study. Thus, the two formulations were not only interconvertible but also safe and well-tolerated. Furthermore, *de novo* administration of liquid *versus* tablet formulations showed equal immunosuppressive benefits [53].

5. Clinical efficacy

5.1 Phase I study in stable renal transplant patients

The Phase I study examined the effects of a 2-week course of twice-daily doses of sirolimus on the clinical courses of quiescent renal transplant patients receiving a stable regimen of CsA and steroids. The pharmacokinetic profiles revealed a long $t_{1/2}$ of sirolimus compared with CsA, suggesting that a once-daily dosing regimen would be suitable for future studies of the new agent [54]. Furthermore, because of the lag of 5 - 7 days to achieve adequate trough blood sirolimus concentrations, administration of a 3-fold greater loading dose was recommended to accelerate the therapeutic effects. Although a 2-week course of sirolimus showed neither nephrotoxic nor hypertensive interactions with CsA, sirolimus displayed several dose-dependent adverse effects-thrombocytopenia, granulocytopenia and hyperlipidaemia. Since there was no

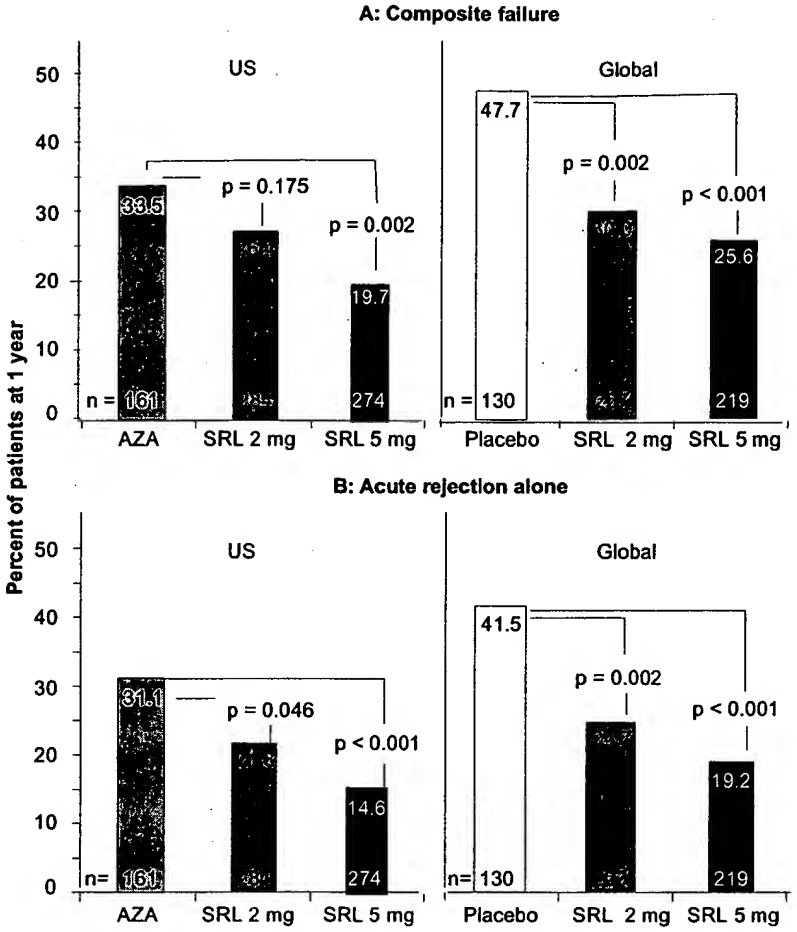

Figure 5. Graft rejection rates. (Panel **A**: composite faliure) One-year data demonstrated that patients treated with sirolimus 2 or 5 mg/day in the pivotal Phase III Global [8] sirolimus trials, and patients treated at the 5 mg/day dose in the pivotal Phase III US trial [7], demonstrated significantly reduced rates of efficacy failure (a composite of the occurrences of acute rejection episodes, graft loss, and/or death); (Panel **B**: acute rejection alone) both trials showed a significant reduction in the occurrence of acute rejection episodes than among patients treated with either placebo (n = 130) or Aza (n = 161) at both sirolimus doses. AZA: Azathioprine; SRL: Sirolimus.

apparent exacerbation of CsA side effects other than hypercholesterolaemia, the sirolimus-CsA combination seemed suitable for further clinical development.

5.2 Initial Phase II studies

The first Phase I/II open-label, ascending-dose trial in non-identical, living-related donor renal transplant recipients treated with the combination of sirolimus and CsA-Pred revealed a reduced incidence of acute rejection episodes (from 32 to 7.5%). Furthermore, withdrawal of steroid therapy was tolerated by most patients as early as at 1 week [55]. The initial observation that sirolimus and CsA acted in a synergistic fashion in humans was the similar 12% incidence of acute rejection episodes among non-African-American patients, whether they received full or reduced exposures to the oil-based CsA formulation in a multi-centre Phase II trial [56].

An independent path of study during Phase II trials assessed the activity of sirolimus as base therapy without CsA but rather in combination with Aza [5] MMF [6] or Pred. All arms showed about equal, 40% incidences of treated acute rejection episodes within the first 6 months. Although the renal function at 6 and 12 months was significantly better in the sirolimus arms, the 40% incidence of acute rejection episodes was of concern, since these episodes not only predispose to the occurrence of chronic rejection but also engender increased costs for diagnosis and treatment.

All sets of Phase II studies provided greater insight into the side effect profile of sirolimus. Increased serum triglyceride and, to a lesser extent, cholesterol values were observed by the third month but were responsive to conventional therapy with fibrates and statins, respectively. Dose-dependent and reversible reductions in the platelet and white blood cell counts tended to be seen during the first month. Finally, a slower recovery of the haemoglobin value upon reversal of

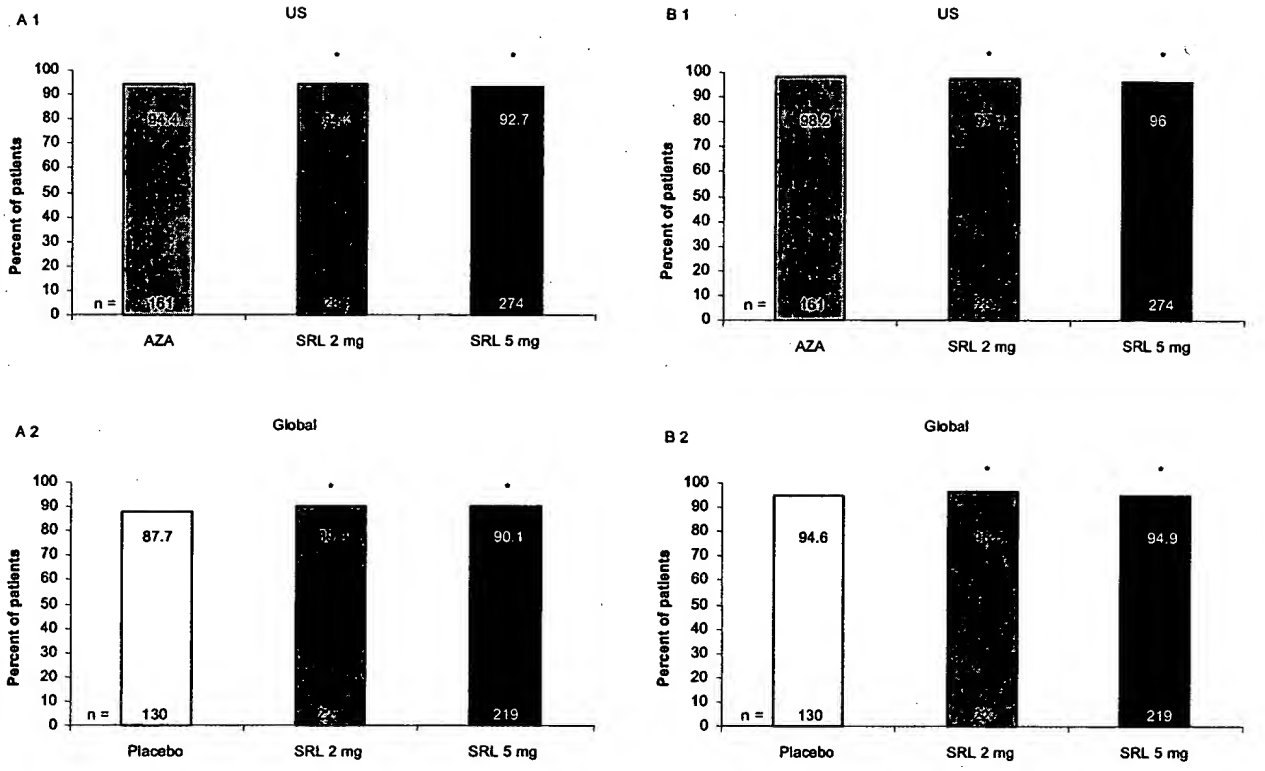

Figure 6. Percentage of grafts (A) and patients (B) surviving at 1 year. * None of the differences are statistically significant.

chronic renal failure was experienced by patients undergoing sirolimus-CsA treatment, compared to CsA-only regimens. These events appeared to correlate with trough concentrations > 15 ng/ml [55], confirming the utility of therapeutic drug monitoring. The spectrum and severity of adverse events, however, seemed mild when compared with the potency of the drug combination to reduce the incidence of acute rejection episodes and to facilitate steroid withdrawal.

5.3 Phase III Studies

Pivotal Phase III US [7] and global [8] trials showed that the 6-month composite rates of efficacy failure (the occurrences of acute rejection episodes, graft loss and/or death) were significantly reduced among patients who received sirolimus 2 or 5 mg/day *versus* placebo or Aza, in addition to CsA-Pred treatment. At 1 year, both sirolimus groups displayed a benefit compared to placebo (global trial) and in the US trial, only the 5 mg/day sirolimus dose showed greater efficacy than Aza (Figure 5a). However, a significant benefit (reduction in the occurrence of acute rejection episodes, the major component of the composite failure rate) was noted in both trials for the two sirolimus doses at both 6 and 12 months (Figure 5b). Among these episodes there was a lower incidence of events that were graded either as moderate or severe by the Banff system, or required antibody therapy for rejection reversal. As is the case for other immunosuppressive agents, larger (5 mg/day) doses of sirolimus seemed to be necessary in high-risk recipients of African-American ethnicity, of cadaveric donor organs, or of grafts carrying > 3 HLA mismatches [7]. Due to advances in the treatment of rejection, only 4 - 8% of all grafts were lost. Therefore, one could not discern whether sirolimus improved transplant survival rates (Figure 6a). Furthermore, at 1 year, despite the enhanced immunosuppression, patient survival rates were similar, probably related to the fact that recipient death was usually due to non-transplant related causes (Figure 6b). Using intent-to-treat methodology, which includes all patients entered into the trial regardless of whether they continued under sirolimus treatment, there was a modest erosion of immunosuppressive benefits at 2 years, most likely due to premature termination of sirolimus therapy [57].

The synergistic nature of the interactions between sirolimus and CsA were documented in humans by a retrospective median effect analysis [58] using concentration data from blood samples obtained from subjects in the pivotal Phase III multi-centre trials [9]. The analysis revealed a combination index (CI) value of 0.65, wherein CI = 1.0 represents an additive interaction, CI > 1 an antagonistic interaction and CI < 1.0, a synergistic interaction.

Although patients in the sirolimus-CsA arms did not dis-

play an increased incidence of infectious or malignant complications, they did experience a range of non-immune toxicities, including potentiation of some CsA-related adverse reactions, particularly kidney dysfunction. To explore the renal effects, salt-depleted rats, which display a nephrotoxic injury akin to that observed in humans [59], were treated with combinations of sirolimus and CsA. Animals receiving sirolimus displayed markedly augmented intrarenal CsA concentrations that were disproportionate to those observed in rats administered only CsA at the same doses. These disproportionate elevations correlated with the reductions in renal function. Indeed, when the data were analyzed based on intrarenal CsA concentrations rather than on the CsA dose, sirolimus was shown to actually mitigate the nephrotoxic injury that would have otherwise been anticipated had the animal received a sufficient dose of CsA to produce the observed intrarenal exposure to CsA [60].

Administration of sirolimus alone has been shown to be free of intrinsic nephrotoxicity both in animal models [61] and in clinical trials. The renal function of psoriatic patients was not changed from the baseline value after treatment for 3 months with sirolimus [41]. Furthermore, allograft renal function was significantly better among patients treated with sirolimus in combination with Aza or MMF and Pred than among those treated with CsA-Aza-Pred [5,6]. Thus, renal function may be better preserved if relatively higher exposures of the non-nephrotoxic agent sirolimus are combined with reduced doses of the nephrotoxic agent CsA, thereby not only exploiting the synergistic immunosuppressive interactions [9,62] but also minimising potentially nephrotoxic pharmacokinetic interactions. However, this strategy must be tailored to the individual patient since other adverse effects, such as diarrhoea, hyperlipidaemia and thrombocytopenia, show direct relations to sirolimus dose and concentrations. These findings suggest that concentration-control algorithms must be developed to minimise the toxicities and optimise the TW of sirolimus-CsA regimens.

6. Other therapeutic applications of sirolimus in renal transplant patients

6.1 CsA elimination

A beneficial effect of the sirolimus-CsA combination of reducing the incidence of acute rejection episodes was observed. This occurred primarily during the first 90 days post-transplant and due to the potential long-term nephrotoxicity of CsA, two clinical trials assessed the benefits of elimination of the calcineurin antagonist after this critical period. Patients withdrawn from CsA treatment experienced significantly fewer treatment-emergent adverse events compared to those not withdrawn from CsA. Graft and patient survival rates were similar and the incidence of acute rejection episodes was not significantly increased [63,64]. While these studies documented better renal function in the CsA elimination arm, it should be noted that patients in this cohort were treated *de novo* with lower CsA exposures, which by itself might have produced the effect. Furthermore, there were more acute rejection episodes post-CsA withdrawal than among patients maintained on the sirolimus-CsA combination. Since the numbers of patients was small, the study was not adequately powered to see a significant difference in the incidence of rebound rejection episodes. Despite these reservations, sirolimus may play an important role in some patients by eliminating the nephrotoxic injury caused or exacerbated by calcineurin antagonists, the indication for drug approval by the European Committee on Proprietary Medicinal Products.

6.2 Refractory rejection

The addition of sirolimus to a CsA-based regimen has been shown to control the unrelenting progression of acute rejection processes refractory to treatment with antilymphocyte antibodies [65]. Although acute rejection episodes represent an important complication following renal transplantation, more than 80% of these episodes are reversed by available treatment regimens, namely high-dose corticosteroids with or without polyclonal antibodies (pAbs) or mAbs. Immune processes refractory to these agents almost inevitably progress to allograft loss. In an attempt to minimise graft loss, sirolimus was administered at a dose of 7 mg/m^2 for 5 days and thereafter at 5 mg/m^2 in combination with only modestly reduced doses of CsA and initially baseline amounts of steroids that were either tapered or withdrawn as tolerated. Allograft rejection episodes refractory to OKT3 and/or to MMF were reversed in 96% of 24 renal transplant recipients, which was a significantly better result than that achieved with MMF [66]. However, the trend toward a greater, albeit insignificant, increase in the mortality rate among the sirolimus cohort compared with the MMF group indicates the need for further study to develop an optimal regimen.

6.3 African-American renal transplant recipients

This ethnic group shows a proclivity to a greater incidence and severity of rejection episodes and graft loss than other patients. In the pivotal US multi-centre trial, African-American patients who received either 2 or 5 mg/day sirolimus failed to show a significant reduction in acute rejection prophylaxis compared with the CsA-Aza-Pred regimen [7]. However, a subsequent single-centre study utilising higher drug doses (5 - 15 mg/day) and target trough concentrations (15 ± 5 ng/ml) of sirolimus in combination with a CsA-Pred regimen demonstrated significantly better rejection prophylaxis than in the pivotal trial. The 19.2% incidence of biopsy-proven acute rejection episodes at 2 years after kidney transplantation among patients treated with sirolimus-CsA-Pred was significantly lower than the 43.3% incidence among African-Americans treated with a CsA-Pred dual drug combination ($p = 0.004$) [67]. Not only were patient and graft survivals not compromised but also the regimen of increased drug exposure engendered fewer side effects than had been seen with non-African-American patients treated with a similar protocol [67].

Despite the augmented sirolimus exposure, African-Americans showed little change in serum cholesterol, triglyceride, or creatinine values and/or red or white blood cell, or platelet counts. The tolerance of African-American patients to high sirolimus exposures in combination with CsA contrasts with the markedly adverse experience of an increased incidence of post-transplant diabetes mellitus displayed by this ethnic group when treated with tacrolimus alone [68] or in combination with sirolimus. Thus, in the preliminary study of Anton Haney [69], African-American recipients converted from steroids to sirolimus displayed a 40% incidence of *de novo* diabetes mellitus, as well as inferior outcomes compared with patients on a steroid-free CsA-sirolimus regimen [70].

6.4 Sirolimus use in combination with tacrolimus

Although initial *in vitro* studies suggested a pharmacodynamic antagonism between sirolimus and tacrolimus, possibly due to their competition for FKBP 12 in the cytoplasm, this drug combination has been subjected to preclinical studies [71] and to early clinical use. The beneficial results with the combination of sirolimus-tacrolimus-steroids in liver transplantation described in a published letter have been questioned because not all episodes that were treated with increased steroid doses were reported as rejections, and because the authors did not obtain the tissue necessary to establish these treated episodes as biopsy-proven, their index of insufficient immunosuppression [72]. Clinical multi-centre trials of the tacrolimus-sirolimus-steroid combination in liver transplantation have been abandoned due to an increased incidence of hepatic artery thrombosis. In this author's experience in kidney transplantation, the use of a tacrolimus-sirolimus-steroid combination leads to a greater incidence of haemolytic uraemic syndrome than a CsA-sirolimus-steroid combination [73]. Considerably greater success seems to have been experienced by the Edmonton Group using a steroid-avoidance protocol including humanised anti-IL-2R mAbs (daclizumab, Zenapax®, Roche, Nutley, NJ, USA) with tacrolimus, sirolimus and MMF for pancreatic islet transplantation [74]. However, it is unclear which agent is critical to the regimen and whether any of the drugs acts in more than an additive fashion. Although the 1-year outcomes are superb, the attrition noted in the second year suggests that 5-year follow-up will be necessary to ascertain whether the benefits outweigh the morbidities of the intense immunosuppression.

6.5 Induction strategies for delayed graft function

Patients whose renal transplants display delayed graft function (DGF) are predisposed to an increased occurrence of acute rejection episodes and to attenuated graft survival [75,76]. Unfortunately, this complication has recently increased in frequency in the US owing both to the use of marginal donor organs and to the increasing recognition that adequate calcineurin antagonist concentrations (in the absence of sirolimus) are necessary to avert early allograft rejection [77]. In the multi-centre pivotal trials, *de novo* treatment with sirolimus-CsA combinations did not further increase the incidences of rejection or graft failure for DGF patients compared with the rates among the CsA plus Aza/placebo cohorts who experienced this complication.

To avert the use of potent but toxic antilymphocyte preparations, which may produce the cytokine release syndrome [78], nephropathy [79] and/or hypersensitivity reactions, a chimeric (c-) anti-IL-2R mAb (basiliximab, Simulect®, Novartis, Basel, Switzerland) has been advocated for induction immunosuppression. In a pivotal US randomised, blinded, placebo-controlled clinical trial, patients treated with c-anti-IL-2R mAb showed a significantly higher incidence of urine production in the operating room ($p = 0.030$) and an almost significant ($p = 0.07$) reduction in the incidence of DGF compared to placebo-treated recipients [80]. Furthermore, over a 1-year follow-up period the mean serum creatinine and calculated creatinine clearance values of patients treated with basiliximab were consistently and significantly better than those of the group receiving placebo. Finally, the combination of basiliximab with full exposure to CsA reduced the incidence of acute rejection episodes by about 30% [80,81].

A novel strategy for induction therapy combined sirolimus with c-anti-IL-2R-mAb, allowing a prolonged window of up to 2.5 months of freedom from calcineurin antagonist therapy; thereby facilitating recovery from DGF. After a pilot study showed the feasibility of this strategy [82], a formal study [65] confirmed the principle using three contemporaneous but nonrandomised cohorts: immediate-function patients treated *de novo* with CsA-c-IL-2R mAb-Pred ($n = 21$; group II) and DGF patients treated with either antilymphocyte preparations (Pred and CsA initiated at 7 - 14 days ($n = 18$; group III)) or with sirolimus-c-anti-IL-2R mAb-Pred and inception of CsA once the serum creatinine value was ≤ 2.5 mg/dl ($n = 43$; group I). The combination of sirolimus and c-anti-IL-2R mAbs provided the lowest rate of acute rejection episodes, not only among all (16%) but particularly among low-risk transplant recipients (8%). A subsequent study revealed that high-risk African-American or re-transplant recipients were able to realise similar benefits if sirolimus was combined with polyclonal anti-lymphocyte preparations rather than c-anti-IL-2R mAbs [83]. Thus, the use of sirolimus to tailor the induction regimen to compensate for DGF may facilitate early renal recovery from ischaemia-reperfusion injuries.

6.6 Steroid withdrawal

The myriad of complications related to chronic steroid treatment has led to a number of trials attempting steroid withdrawal from CsA or tacrolimus regimens, yielding acute rebound rejection rates within 90 days of 15 - 51% [84]. However, among 131 patients (18 African-American; 36 Hispanic; 63 Caucasian; and 14 others) receiving sirolimus-CsA combination therapy the long-term results of steroid withdrawal have been more favourable [70]. Patients were tapered off ster-

oids 5 - 1220 days post-transplant (median 415 days) and sirolimus doses were adjusted to achieve 5 - 15 ng/ml trough (C_0) concentrations by an HPLC/UV assay. Only 16 patients (12.1%) required re-institution of steroids due to immunologic causes: acute rejection in six patients (4.5%) and chronic rejection in 10 patients (7.6%), five of whom lost their grafts. The mean serum creatinine value as well as blood pressure, triglycerides and cholesterol levels were not significantly different from those at the time of withdrawal (1.8 ± 0.9 mg/dl). However, the rate of acute rejection episodes was higher among the 39 patients withdrawn before 6 months (median 35 days) *versus* the 92 patients withdrawn thereafter (median 502 days; 10.2 *vs.* 2.1%, respectively, p = 0.06). Since this 10.2% rate is much lower than the 35% rate seen in previous studies using calcineurin antagonists alone, steroid withdrawal to a sirolimus-CsA regimen seems to proffer a safe maintenance regimen for renal transplant recipients.

7. Safety and tolerability

7.1 Hyperlipidaemia

Hyperlipidaemia is the most serious adverse effect of sirolimus treatment, potentially exacerbating CsA-induced hypercholesterolaemia, steroid-induced hypertriglyceridaemia and the dyslipidaemias associated with renal disease as well [85]. Metabolic studies suggest that sirolimus treatment delays the clearance of circulating, low, intermediate and very-low-density lipoproteins as well as their remnants [86]. Hyperlipidaemia occurs in about 40% of renal transplant recipients under sirolimus treatment, reaching maximum values during the second or third month post-transplant and appearing to be drug dose- and concentration-dependent [87]. However, our experience suggests that, in the majority of patients, this complication is readily controlled by counter-measure therapy. A low-fat diet, increased exercise and withdrawal from Pred therapy frequently ameliorate the condition, but patients with triglyceride or cholesterol values above 300 or 200 mg/dl, respectively, clearly require counter-measure therapy. Interestingly, in the US Phase III sirolimus trial, at both 1 and 2 years, no significant difference in the incidence rates of conditions attributed to elevated blood lipids - pancreatitis, myocardial infarction, or stroke - was observed between patients treated with sirolimus 2 or 5 mg daily *versus* Aza or placebo [7].

7.2 Bone marrow suppression

Many immunosuppressive regimens are associated with myelosuppression, particularly in the first few months after renal transplantation [88]. Multiple factors may exacerbate the problem: namely, intra-operative blood loss stimulating the hyporesponsive bone marrow of uraemic patients, post-operative infections and/or alloimmune reactions to the graft. Although the condition is more common in patients treated with nucleoside synthesis inhibitors or polyclonal antilymphocyte antibodies [89], a reversible, concentration-dependent syndrome of myelosuppression occurs among 61% of sirolimus-treated patients, particularly those with $Cmin_{ss}$ values ≥ 16 ng/ml during the first 4 weeks of therapy [88]. The myelosuppression may be related to blockade of critical cytokine signals that promote maturation and/or proliferation of bone marrow elements, including IL-11 on platelets, granulocyte colony stimulating factor on leukocytes and erythropoietin on red blood cell precursors. During the early post-transplant phase, myelosuppression may provide a convenient index of sirolimus toxicity, since it tends to occur during the first month (as opposed to hyperlipidaemia at 2 - 3 months) and since it, in contradistinction to hyperlipidaemia, is not produced by CsA, steroids, or dietary indiscretions. However, sirolimus-induced myelosuppression may be obfuscated by concomitant treatment with the nucleoside synthesis inhibitors Aza or MMF.

8. Expert opinion

The introduction of sirolimus represents the greatest advance in immunosuppressive therapy since CsA. Sirolimus not only spares elements of nonspecific host resistance but also synergistically promotes the immunosuppressive effect of the calcineurin antagonist CsA, as shown by the Phase II data and, more particularly, the median effect analysis of the combined US and global Phase III pivotal studies. This pharmacodynamic effect permits a reduction in CsA exposure by 60%, which is in addition to the 15 - 25% dose reduction resulting from the pharmacokinetic interaction with sirolimus [89]. However, concentration-controlled studies will be critical to refining the use of the drug combination by discovering the optimal ratio of exposures to the two agents. Forsaking the benefit of synergistic interactions, 40% of patients treated in Phase II studies using sirolimus as the base agent in combination with Pred and either Aza [5] or MMF [6] experienced treated acute rejection episodes. The additional cost of about US$5000 per episode for medical care to diagnose and reverse the event, as well as the association of occurrence of an acute rejection episode with subsequent appearance of chronic rejection, suggest to this author that combination therapy with sirolimus and CsA represents a more efficacious and cost-effective approach than elimination of CsA.

In view of these unique therapeutic opportunities, it is difficult to understand why sirolimus has only had slow market penetration. Although the value of concentration monitoring to enhance the outcomes of sirolimus immunosuppression was not initially recognised in the FDA guidelines, transplant physicians have become accustomed to using drug measurements to guide therapy. Since an automated immunoassay produced by Abbott [40] was never introduced into the market, the HPLC/UV and HPLC/mass spectrometry (MS)

methods, which have been clinically validated [34,90,91], were the only reference technologies. However, both methods require sophisticated technology to produce reliable results within the range of determination (C_0 = 2 - 50 ng/ml). The difficulty in obtaining concentration measurements at many transplant centers has clearly retarded market penetration of this potent agent. In contrast, the lack of automated techniques for measurement did not represent a disincentive to the introduction of the relatively weak nucleoside synthesis inhibitor MMF, albeit that drug concentrations are now recognised as important to optimise outcomes with this agent [92]. Thus, physicians recognised that overdosing of the potent agent sirolimus could be hazardous, whereas MMF might be used with less morbidity.

Although HPLC/UV trough values ≥ 15 ng/ml have been shown to correlate with the occurrence of adverse reactions [55], the guidelines for sirolimus concentrations are based upon the immunosuppressive matrix. Recipients treated *de novo* with only a 30% reduction in CsA exposure require sirolimus C_0 levels of ≥ 5 ng/ml [55]. In contrast, sirolimus C_0 values ≥ 10 ng/ml are required when the CsA exposure has been reduced by 60% [89] and ≥ 20 ng/ml when sirolimus forms the base therapy [5].

It is increasingly recognised that the major cause of long-term renal transplant loss is patient death, primarily due to arteriosclerotic cardiovascular disease. Thus, there have been concerns about the impact of the hyperlipidaemic effects of high doses of sirolimus to enhance the hypercholesterolaemia produced by CsA. However, the 2-year data from the pivotal studies do not reveal an increased risk of death due to this cause among sirolimus-CsA-Pred compared with CsA-Aza-Pred or CsA-placebo-Pred patients. Two advances may mitigate the problem of hyperlipidaemia. Firstly, there is increasing evidence that prophylactic treatment with statins proffer potential benefits to all renal transplant recipients due to their lipid-lowering and their possible immunosuppressive effects [93]. Secondly, it seems to be possible to delineate pre- and post-transplant demographic features that represent risk factors for hyperlipidaemic side effects: diabetic recipients are more and African-American patients less frequently affected by sirolimus therapy.

In contrast, five observations suggest that sirolimus therapy may reduce the occurrence and/or slow the progression of vascular disease in transplants including chronic rejection [94]. Firstly, by permitting minimisation or elimination of calcineurin antagonists, the use of sirolimus may mitigate the nephrotoxic injury. Secondly, by inhibiting growth factor-driven proliferation of endothelial and smooth muscle cells as documented using *in vitro* systems [95-101], sirolimus may attenuate the immuno-obliterative arterial and arteriolar pathophysiological processes that are hallmarks of chronic rejection. Thirdly, in similar fashion to the dampening of responses provoked by balloon catheter injury to vascular walls [102], or generated by alloimmune reactions toward aortic grafts in animals [103], or elicited by stents in human coronary arteries [104], sirolimus may protect against vasculopathic lesions in transplants. Fourth, in parallel to the effect of sirolimus to mitigate chronic rejection in rat models [105], the drug may directly block the generation of critical immune mediators [106]. Finally, by markedly reducing the incidence of acute rejection episodes, sirolimus may lower the risk of chronic rejection [107,108]. Clearly, inhibitory effects of the drug on immune and non-immune mediators of vasculopathic injuries have the potential to engender wide-ranging benefits in medical therapy. During the past decade of using sirolimus in 650 renal transplant patients, the agent has come to play an increasing role in this author's protocols for clinical immunosuppression. However, we have not yet approached the horizon of optimal therapy.

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